Short Communication

Detection of a Novel Recombinant Norovirus from Sewage Water in Toyama Prefecture, Japan

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SUMMARY: Recently, the recombination event of norovirus (NoV) has been reported with high frequency, suggesting that RNA recombination is a major driving force in NoV evolution. To assess the incidence of NoV recombination in a residential area, we conducted a molecular biological survey of NoVs existing in sewage water in Toyama Prefecture, Japan. Although GII/4 was predominantly detected in sewage water that was associated with a high frequency of outbreaks caused by this genotype, other genotypes, including two types of recombinant strain, were identified during the survey period. One of the recombinants is the WUG1 type, which was first detected in Saitama Prefecture in 2000. The other recombinant is a novel type derived from two parent strains of genogroup II, GII/7 for the RNA-dependent RNA polymerase and GII/13 for the capsid. This suggests that certain NoVs circulating in the area are occasionally changing their genetic properties by recombination events.

Norovirus (NoV), a member of the *Caliciviridae* family, is a major causative agent of acute gastroenteritis in human. Over the last decade, NoV-associated gastroenteritis has vigorously expanded worldwide and has been recognized as a public health problem (1,2). Recent developments in molecular biological techniques to detect the NoV genome contributed to an acquisition of large numbers of NoV strains (3-5). These NoVs are now classified into five genogroups (GI to GV) by molecular characterization based on the partial or complete capsid, or according to their RNA-dependent RNA polymerase (RdRp) sequences (3,6-8). GI, GII and GIV are known to infect humans (9). GII, the most prevalent causal genogroup in recent acute gastroenteritis, contains porcine strains as well as human strains. GIII and GV are composed of bovine and murine strains, respectively (5). GI and GII are further divided into many genotypes, and this classification reflects constant evolution with the discovery of new strains.

For most NoV strains, sequence information on the RdRp region obtained by diagnostic reverse transcription (RT)-PCR correlates with clustering based on the capsid sequence. However, several studies have shown that the clusters to which strains belong seem to depend on either the RdRp or capsid gene analyzed. This suggests the occurrence of recombination among NoV strains (4,6,10-13). A growing number of reports on NoV recombinant strains suggest that RNA recombination is a critical driving force in NoV evolution.

Raw sewage water is a suitable material for capturing NoVs circulating in the environment, because such water could contain enteric viruses shed by infected people. Especially, analyses of raw sewage show that they can detect NoVs shed by asymptomatic populations that have not exhibited clinically recognized NoV infections (14-16). In the present study,

we conducted a molecular biological survey of NoVs existing in sewage water in Toyama Prefecture, Japan, to learn the incidence of NoV recombination in this area.

Raw sewage samples collected monthly from October 2006 to December 2007 were used in this examination. Raw sewage water was collected at a point of influx to a primary treatment tank at a sewage disposal facility in the western Toyama Prefecture. This facility covers an area having about 300,000 inhabitants. Two liters of collected raw sewage was centrifuged at 3,000 rpm for 30 min, and 1 liter of each supernatant was subsequently 100-fold concentrated to a 10-ml volume by the filter adsorption/elution method or polyethylene glycol (PEG) precipitation method, as described elsewhere (16-18). Viral RNA was extracted from 140 μl of concentrated raw sewage using a QIAamp viral RNA Mini Kit (Qiagen K.K., Tokyo, Japan) according to the manufacturer's instructions. Extracted RNA was treated with 5U of DNase I (Takara Bio Inc., Shiga, Japan), and cDNA was synthesized by SuperScript III reverse transcriptase (Invitrogen Japan K.K., Tokyo, Japan) with a random hexamer according to the manufacturer's instructions. The cDNA obtained was used for the following PCR. To screen the recombinant NoV, primers 1421f (5′-ATA CCACTATGATGCAGAYTA-3′), 1364f (5′-YTCYTTCTAT GGYGATGATGA-3′), G1SKR (5′-CCAACCCARCCAT TRTACA-3′), G2SKR (5′-CCRCCNGCATRHCCRTTRTA CAT-3′) and NV2oR (5′-GTRAACGCRTTYCCMGC-3′) (1,15,19) were chosen. 1421f and 1364f are positioned at nt 4279-4299 and 4585-4605 on the RdRp gene of the NoV GII Loadsdale strain (GenBank accession no. X86557), respectively. Our preliminary study confirmed the cross-reactivity of these primers with corresponding sites of NoV GI strains. G1SKR binds as a negative sense at nt 5653-5671 on the capsid gene of the NoV GI Norwalk virus (GenBank accession no. M87661). G2SKR and NV2oR bind as a negative sense at nt 5367-5389 and 5412-5428 in the capsid gene of the NoV GII Loadsdale strain, respectively. A primer pair of 1421f and G1SKR was used for the first PCR, and a pair of 1364f and G1SKR was used for semi-nested PCR to detect NoV GI strains. To detect NoV GII, the first round of PCR

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using a primer pair of 1421f and NV2oR, followed by nested PCR using a primer pair of 1364f and G2SKR, was performed. The obtained PCR products, composed of the 3′ end of RdRp and the 5′ part of capsid genes, were cloned into pGEM-T Easy Vector (Promega K.K., Tokyo, Japan) according to the manufacturer's instructions. Subsequently, nucleotide sequences of amplified fragments were determined. The nucleotide sequences of the 3′ end of the RdRp gene and the 5′ part of the capsid gene of the analyzed strains were separately applied for the construction of the phylogenetic trees to screen the recombinant type of NoV. Multiple sequence alignments of NoVs were generated by Clustal W, and the bootstrapped phylogenetic trees were constructed by the neighbor-joining method, with 1,000 bootstrap replicates in the Molecular Evolutionary Genetics Analysis version 3.1 (MEGA 3.1) software. The genetic distances among NoV strains were calculated by Kimura's 2-parameter method (20). The genotypes of NoV strains detected in this study were primarily determined according to the classification based on the partial capsid gene as previously proposed by Katayama et al. (4). Since a certain number of reference strains lacked sequence information for the RdRp gene, additional reference strains described in a recent report (13) were quoted to determine the genotypes based on the RdRp gene. The designation GIIb, GIIc and GIId were used to describe the genetic clusters composed of genetically diverse RdRp genes (13,21) in the phylogenetic tree constructed in this study.

We previously reported that NoVs could be detected in raw sewage water throughout the year (16). That report also indicated the existence of multiple NoV strains in samples collected at the same time. On the basis of our previous report, the present study adopted the molecular cloning strategy of the PCR-amplified NoV gene to distinguish multiple strains existing in the samples. As a result, GI/8 strains were detected from the samples collected in September 2006 and in January, March, May and August 2007 (shown as sewage/

Toyama/SW0609-7/06/JP, sewage/Toyama/SW0701-2/07/JP, sewage/Toyama/SW0703-18/07/JP, sewage/Toyama/SW0705-9/07/JP and sewage/Toyama/SW0708-1/07/JP in Fig. 1, respectively) besides the GI/4 strains previously identified (16). Further genetic analysis indicated that these GI/8 strains were a WUG1-type recombinant, which was first detected in Saitama Prefecture in 2000, and composed of the GI/8 capsid gene and the GI/2 RdRp gene (Fig. 1) (4). This WUG1-type recombinant has also been detected in Hiroshima Prefecture as a cause of the outbreak (22). Our study revealed that the WUG1-type recombinant has existed in Toyama Prefecture. These findings suggest that the genetic combination of the GI/2 RdRp and GI/8 capsid genes was stable in the environment and widely prevalent in Japan.

One strain forming a new branch and two strains clustering with the GI/7 Winchester strain were also detected in February and March 2007 (shown as sewage/Toyama/SW0702-1/07/JP, sewage/Toyama/SW0702-11/07/JP and sewage/Toyama/SW0703-10/07/JP in Fig. 1, respectively). These strains probably possessed the indigenous RdRp gene, although this gene was not clearly identified because of insufficient reference data concerning the RdRp gene of NoV GI (Fig. 1).

As for the GII strains, GII/4 was dominantly detected. This seemed to correlate with the high frequency of outbreaks caused by this genotype. GII/6 strains were also detected in January and May 2007 (shown as sewage/Toyama/SW0701-35/07/JP and sewage/Toyama/SW0705-2/07/JP in Fig. 2, respectively). These strains were not recombinant, since the clustering based on the RdRp gene was concordant with the capsid sequences (Fig. 2). Notably, GII/13 strains were detected in March and September 2007 (shown as sewage/Toyama/SW0703-6/07/JP and sewage/Toyama/SW0709-11/07/JP in Fig. 2, respectively). Construction of the phylogenetic tree based on the capsid gene revealed that both strains belonged to the same cluster. However, another phylogenetic
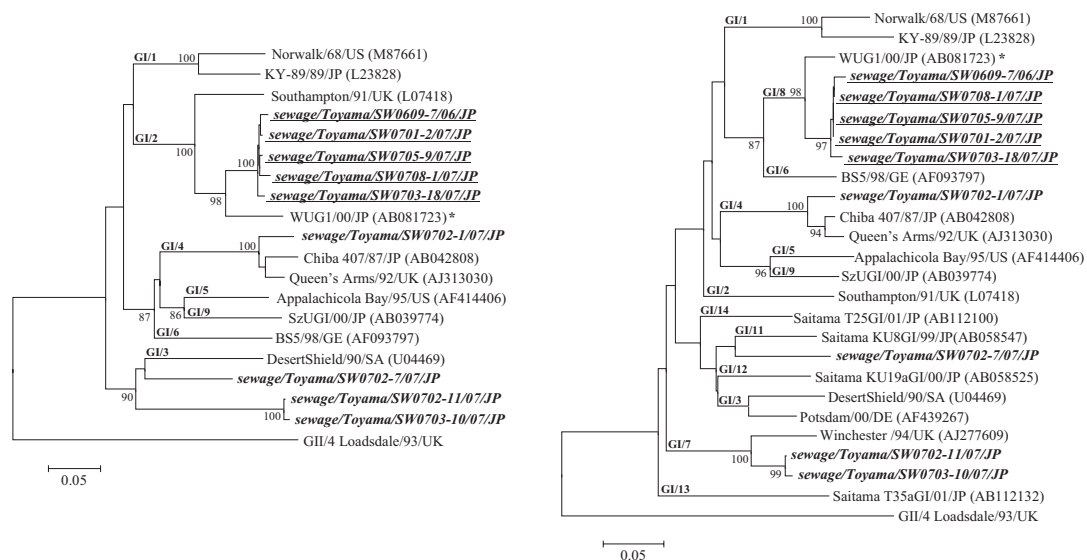

Fig. 1. Phylogenetic analysis of NoV GI strains. The left tree is reconstructed for the 3′ end of the RdRp region. The right tree is reconstructed for the 5′ part of the capsid region. The DDBJ accession numbers of each reference strain are shown in the parentheses. Asterisk indicates a recombinant strain previously reported (4). More than 80% of bootstrap values are shown on the branches. Newly sequenced strains are shown as an italic face, and suspected recombinant strains are underlined. The nucleotide sequence data of sewage/Toyama/SW0609-7/06/JP, sewage/Toyama/SW0701-2/07/JP, sewage/Toyama/SW0702-1/07/JP, sewage/Toyama/SW0702-7/07/JP, sewage/Toyama/SW0702-11/07/JP, sewage/Toyama/SW0703-10/07/JP, sewage/Toyama/SW0703-18/07/JP, sewage/Toyama/SW0705-9/07/JP and sewage/Toyama/SW0708-1/07/JP are deposited in the DDBJ database under accession numbers of AB504688, AB504692, AB504695, AB504697, AB504698, AB504701, AB504702, AB504705 and AB504708, respectively.
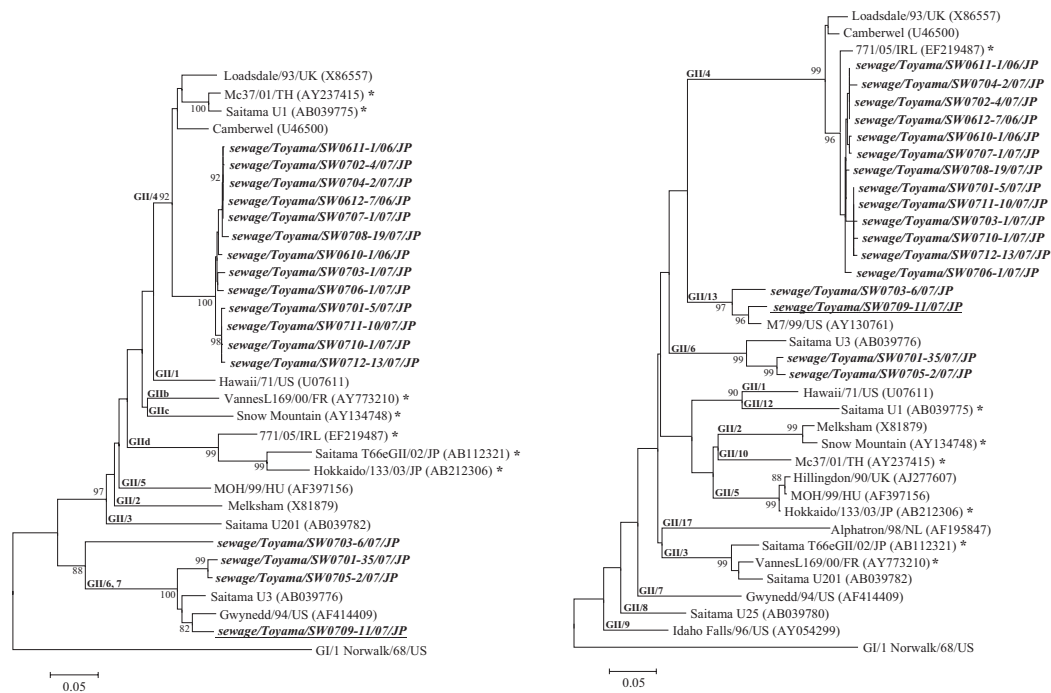
Fig. 2. Phylogenetic analysis of NoV GII strains. The left tree is reconstructed for the 3′ end of the RdRp region. The right tree is reconstructed for the 5′ part of the capsid region. The DDBJ accession numbers of each reference strain are shown in the parentheses. Asterisk indicates a recombinant strain previously reported (13). More than 80% of bootstrap values are shown on the branches. Newly sequenced strains are shown as an italic face, and suspected recombinant strains are underlined. The nucleotide sequence data of sewage/Toyama/SW0610-1/06/JP, sewage/Toyama/SW0611-1/06/JP, sewage/Toyama/SW0612-7/06/JP, sewage/Toyama/SW0701-5/07/JP, sewage/Toyama/SW0701-35/07/JP, sewage/Toyama/SW0702-4/07/JP, sewage/Toyama/SW0703-1/07/JP, sewage/Toyama/SW0703-6/07/JP, sewage/Toyama/SW0704-2/07/JP, sewage/Toyama/SW0705-2/07/JP, sewage/Toyama/SW0706-1/07/JP, sewage/Toyama/SW0707-1/07/JP, sewage/Toyama/SW0708-19/07/JP, sewage/Toyama/SW0709-11/07/JP, sewage/Toyama/SW0710-1/07/JP, sewage/Toyama/SW0711-10/07/JP and sewage/Toyama/SW0712-13/07/JP are deposited in the DDBJ database under accession numbers of AB504689, AB504690, AB504691, AB504693, AB504694, AB504696, AB504699, AB504700, AB504703, AB504704, AB504706, AB504707, AB504709, AB504710, AB504711, AB504712 and AB504713, respectively.

tree, based on their RdRp gene, showed that sewage/Toyama/SW0709-11/07/JP formed a cluster with the GII/7 Gwynedd strain, whereas sewage/Toyama/SW0703-6/07/JP branched outside of the cluster containing GII/6 and GII/7 strains (Fig. 2). SimPlot (ver. 3.5.1, provided by SCRoftware website) (23) analysis showed more than 90% similarity between sewage/Toyama/SW0709-11/07/JP and the GII/7 Gwynedd strain located at the 3′ end of the RdRp gene, whereas the similarity was drastically reduced rearward of the junction site between the *orf1* and *orf2* genes, which had been reported as the most common breakpoint among recombinant NoVs (24) (Fig. 3A). In addition, the similarity between the sewage/Toyama/SW0709-11/07/JP and GII/13 M7/99/US strains at the 5′ part of the capsid gene was more than 95%. These results indicate that sewage/Toyama/SW0709-11/07/JP was the recombinant type derived from the GII/7 ancestor for the RdRp gene and the GII/13 ancestor for the capsid gene. The derivation of sewage/Toyama/SW0703-6/07/JP RdRp gene remains obscure because referential sequence information for the GII/13 RdRp gene is not available. However, the sewage/Toyama/SW0703-6/07/JP RdRp gene has low similarity to that of the GII/6 and GII/7 strains, in contrast with the sewage/Toyama/SW0709-11/07/JP RdRp gene (Figs. 3A and B). The pairwise distance between the GII/6 and sewage/Toyama/SW0703-6/07/JP RdRp genes (0.268-0.280) was correlated to that of capsid genes (0.248-0.261). Therefore, sewage/Toyama/SW0703-6/07/JP seemed to possess an indigenous GII/13 RdRp gene that is thought to be distant from the GII/6 RdRp gene.

Our previous report revealed that healthy infants frequently harbor the GII/7 and GII/13 strains without apparent clinical symptoms, suggesting the virulence of these strains was relatively mild (16). These harbored viruses could be parental strains of the novel recombinant type NoV reported here; such a recombination event potentially influenced their pathogenicity. Indeed, in the winter of 2007 we experienced an outbreak caused by this novel recombinant type NoV at a nursery school (our laboratory's investigation). Various NoVs including GII/7 have been frequently detected from asymptomatic children in Mexico (25). Moreover, a recombinant strain showing a genetic combination similar to that of sewage/Toyama/SW0709-11/07/JP was detected from a hospitalized child with acute gastroenteritis in China (26). These facts suggest that infants and young children are a major reservoir for the genetic and biological diversification of certain NoVs.

Recent epidemiological surveys have revealed that outbreaks associated with recombinant NoVs have sometimes occurred (22,27,28). Recombinant strains having characteristic RdRp have been noted as a cause of outbreaks (27,28). GIIb RdRp, which is genetically distinct from conventional RdRp genes (21), has been shown to recombine with a variety of capsid genes (27). It is noteworthy that different recombinant strains having a common RdRp gene resulted in significant numbers of outbreaks. This fact suggests that a certain type of RdRp may play crucial roles in NoV pathogenicity. Epidemiological and comparative studies of the recombinant NoV strains retrospectively and currently identified may elucidate the significance of genetic recombination.

(A)

SimPlot – Query: sewage/Toyama//SW0709-11/07/JP

- - - **GII/6 Florida**
—— **GII/7 Gwynedd**
········ **GII/4 Loadsdale**

(B)

SimPlot – Query: sewage/Toyama/SW0703-6/07/JP

- - - **GII/6 Florida**
—— **GII/7 Gwynedd**
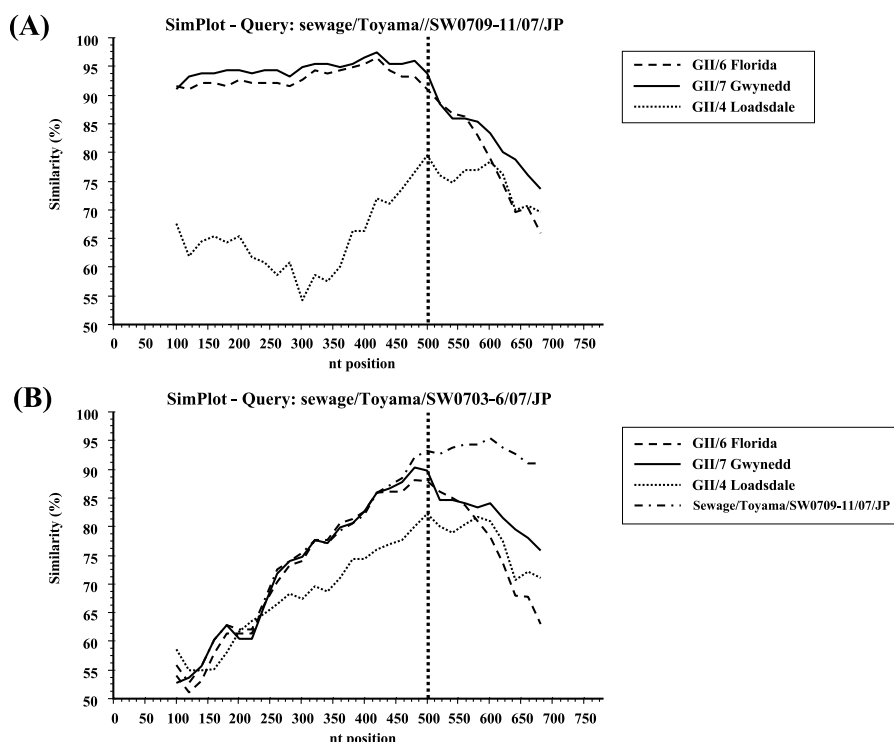········ **GII/4 Loadsdale**
-·-·- **Sewage/Toyama/SW0709-11/07/JP**

Fig. 3. SimPlot analysis for partial RdRp and capsid sequences. The window size was 100 bp with a step size of 10 bp. The query sequences are (A) sewage/Toyama/SW0709-11/07/JP, (B) sewage/Toyama/SW0703-6/07/JP, respectively. The vertical axis indicates the nucleotide identities between the query sequence and reference strains, expressed as percentages. The horizontal axis indicates the nucleotide positions of analyzed fragment. Vertical lines indicate the beginning of the *orf2* gene. Dashed line, GII/6 Frolida; bold line, GII/7 Gwynedd; dotted line, GII/4 (a representative genotype observed in clinical cases) Loadsdale; broken line, GII/13 recombinant sewage/Toyama/SW0709-11/07/JP.

Recently, a recombinant strain derived from different genogroups, GI/3 for the RdRp gene and GII/4 for the capsid gene, were detected from a patient with an acute watery diarrhea in India (29). This finding indicates the potential for further diversification of NoVs by the intergenogroup recombination event. Our study also aimed to examine whether or not intergenogroup recombinants exist in this area. The PCR performed in this study was designed to detect intergenogroup recombinant strains by the cross-binding ability of primers 1421f and 1364f on both GI and GII RdRp genes. Although no evidence of intergenogroup recombination events in this area has been observed, our study does not exclude the possibility that the intergenogroup recombination will occur, since the co-circulation of GI and GII in the environment was revealed by the simultaneous detection of both genogroups from sewage water.

In conclusion, this study detected recombinant NoVs from sewage water, although at a low frequency than that of GII/4, which was a major cause of outbreaks. Certain NoVs were circulating in the area without clinical detection and occasionally changing their genetic properties by recombination events. The molecular cloning strategy adopted in this study encouraged the detection of multiple NoV strains from samples collected at the same time. Genetic analysis of the longer fragment, including the 3′ end of the RdRp gene and the 5′ part of the capsid gene, enabled us to find a recombinant type of NoV. Continuous surveillance with a view toward genetic recombination will promote a precise understanding of the molecular evolution and pathogenicity of NoVs.

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